

Research

Selecting an optimal imaging window for NIR fluorescence wide-field microscopy

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Abstract Near-infrared (NIR) fluorescence imaging has become indispensable in biomedical research owing to its deep penetration and high contrast. Selecting an appropriate imaging window is crucial to maximizing the positive effect of light absorption for achieving the highest signal-to-background ratio. Existing studies on imaging window selection mainly focus on low numerical aperture (NA) macroscopic systems; however, scattered light plays a decisive role in high-NA microscopic systems, thereby leaving a critical gap in current understanding. To address this, we propose the concept of best-matched absorption as a universal criterion for window optimization across diverse imaging conditions in NIR fluorescence wide-field microscopy. By combining Monte Carlo simulations with a custom-built microscope, we systematically quantify how this best-matched absorption coefficient varies with system NA, imaging depth, and probe brightness. Our results reveal that higher NA or elevated probe brightness induces a redshift in the optimal imaging window, which is generalized for both ideal probes and the clinically used indocyanine green. Moreover, we develop tailored window selection strategies for challenging imaging conditions including superficial occlusions, deep background, and targets extending beyond the depth of field. This study establishes a versatile framework for optimizing image quality in NIR fluorescence wide-field microscopy, with direct significance in biomedical applications.

Keywords NIR fluorescence imaging, wide-field microscopy, Monte Carlo method, tissue imaging windows

1. Introduction

In recent years, fluorescence imaging technology utilizing the near-infrared (NIR) spectrum, particularly the second near-infrared (NIR-II, 900–1,880 nm) window, has advanced significantly and been widely applied in areas such as *in vivo* biological imaging [1–6], tumor identification [7–9], and image-guided surgery [10–13]. Compared to visible light and the first near-infrared (NIR-I) region, NIR-II imaging can achieve greater penetration depth and higher signal-to-background ratio (SBR) [1–3,14–16]. Initially, researchers attributed these advantages to reduced tissue scattering and minimal tissue autofluorescence in the NIR-II region [17,18]. However, subsequent studies [19,20] demonstrated that a moderate increase in absorption is also a key factor contributing to the superior imaging performance in this region. Building on this insight, the effects of tissue absorption and scattering on imaging results have been integrated, employing Monte Carlo simulations to establish a method for selecting the optimal imaging window in macroscopic NIR fluorescence imaging under varying probe brightness and imaging depths [21]. Guided by this approach, spectral windows such as NIR-IIx (1,400–1,500 nm) [22], NIR-IIc (1,700–1,880 nm) [23], and even 1,880–2,080 nm [24] have been successfully applied to *in vivo* biological imaging, yielding high-contrast results. This

method has also been adopted for imaging in turbid media [25], where NIR illumination significantly enhances the capability to extract target signals from highly scattering aqueous environments. It is worth noting that these applications are predominantly based on macroscopic photographic imaging systems with relatively low numerical aperture (NA) lenses, where ballistic photons constitute a relatively high portion of the detected signal.

Wide-field microscopy, which offers a large field of view and high resolution, plays an important role in biomedical imaging applications such as the early diagnosis of cerebrovascular diseases [26–29]. Numerous studies have confirmed that combining fluorescence wide-field microscopy with the NIR-II window can achieve deep-tissue, high-contrast microscopic imaging [7,29,30]. Similar to macroscopic imaging systems, wide-field microscopy employs wide-field excitation and area-array detectors for detection. However, it typically uses high-magnification microscope objectives with substantially higher NA to collect fluorescence signals. This high-NA configuration results in a significantly larger proportion of scattered photons in the collected signal. Consequently, wavelengths with higher tissue absorption can exhibit improved image quality, as they preferentially attenuate the longer-path scattered photons. For instance, NIR-II fluorescence wide-field microscopic imaging of mouse cerebral vasculature using imaging windows near 1,450

and 1,600 nm was performed [19], and a higher SBR was observed in the 1,400–1,550 nm region compared to the 1,500–1,700 nm region. Although these studies indicate that moderate tissue absorption positively influences image quality in fluorescence wide-field microscopy, a systematic definition of this “moderate” range and its dependence on imaging conditions remains lacking. It is therefore essential to establish a strategy for selecting the optimal imaging window under different imaging parameters, such as system NA.

In this study, we developed a fluorescence wide-field microscopy system and used indocyanine green (ICG) as a standard fluorescent probe to image patterns generated by a negative contrast resolution target. Based on the Monte Carlo method, we also constructed a simulation model to describe the emission of fluorescence photons from tissue and their subsequent imaging through a wide-field microscope. Both experimental and simulation results demonstrate that the peak signal-to-noise ratio (PSNR) of the image first increases and then decreases with rising tissue absorption coefficient, and there also exists a best-matched absorption coefficient that maximizes the PSNR under given conditions. This coefficient provides a theoretical basis for selecting the imaging window that delivers the best practical performance. We further analyzed how the best-matched absorption coefficient varies with imaging system NA, depth, and probe brightness. Using the actual emission spectrum of ICG, we showed that, as the imaging depth decreased, system NA increased, and the probe brightness improved, the optimal imaging window for ICG redshifted from ~1,200 nm to beyond 1,400 nm. In each case, by switching to the appropriate spectral filter, the imaging PSNR gradually increased. Finally, we investigated the characteristics of the best-matched absorption curve and the corresponding imaging window selection strategy in the presence of superficial signal occlusion, deep defocused background, and targets extending beyond the depth of field (DOF). This work provides a scientific and practical method for selecting the optimal imaging window in NIR fluorescence wide-field microscopy. It helps to dynamically determine the “moderate” level of tissue absorption under specific imaging conditions and is expected to offer valuable theoretical guidance for future applications of NIR fluorescence wide-field microscopy in life science research and clinical practice.

2. Results

2.1 Existence of a best-matched absorption in fluorescence wide-field microscopic imaging

The effect of medium optical parameters on imaging quality was investigated via simulations and *in vitro* phantom imaging, focusing on fluorescence penetration through a scattering medium layer in a fluorescence wide-field microscope system. To this end, we constructed a NIR fluorescence wide-field microscope system employing an area-array detector. A 793 nm continuous-wave laser was used to excite the fluorescence of ICG, and imaging was performed through a negative resolution target overlaid with a liquid medium (Figure 1a and Sect. 5.1). Four objective lenses with varying magnifications and NA were used for

imaging and analysis. In addition, simulations of fluorescence wide-field microscopy were conducted under identical conditions based on the system’s structural parameters. In the simulation model, photons were emitted by a fluorescence source within the tissue. After being absorbed and scattered by the tissue, the emitted photons passed through the objective lens and the tube lens, and were ultimately collected by the detector (Figure 1b and Sect. 5.1).

The *in vitro* phantom imaging results obtained under low and high magnifications are shown in Figure 1c and d, respectively. The reduced scattering coefficient of the medium was fixed at 5 cm^{-1} , while the absorption coefficient gradually increased from 0.18 cm^{-1} to 40 cm^{-1} . When no ink was added, the average absorption coefficient of the medium in the detection wavelength band was 0.18 cm^{-1} , which was the minimum absorption coefficient achievable by the medium. With such a low light absorption coefficient of the medium, even fluorescent photons that undergo multiple scattering within the medium layer can be collected by the camera, resulting in a blurred image. When the absorption coefficient increased to 10 cm^{-1} , the SBR of the image was significantly improved. However, as the absorption coefficient gradually increased to above 20 cm^{-1} and up to 40 cm^{-1} , the image exhibited significant underexposure. The camera integration time was insufficient to meet the exposure conditions, so the SBR of the image decreased, and the dark noise gradually became prominent. We used the image acquired without the addition of liquid medium as the reference image to calculate the PSNR of each set of images under different absorption coefficients. The quantitative evaluation results are shown in Figure 1e and f, respectively. As the absorption coefficient increased, both the simulation and *in vitro* imaging results showed a trend of the PSNR first increasing and then decreasing. Under the condition of $1.25\times$ magnification and $\text{NA} = 0.04$, when the absorption coefficient of the medium was approximately 13 cm^{-1} , the PSNR of the image reached a maximum. At this peak, the absorption of the tissue could maximize the resistance to image blur caused by scattering, and thus was defined as best-matched absorption. When the absorption of the tissue was lower than the best-matched absorption, scattering dominated, and the imaging quality was limited by resolution; whereas when the absorption of the tissue was much higher than this value, an excessively high absorption rate would lead to significant signal loss and insufficient brightness, resulting in underexposure of the image.

When the system magnification was set at $5\times$, and other conditions (including the medium’s absorption and scattering coefficients, excitation power density after the mirror, and camera gain) remained constant, it was observed that images were relatively blurry at low medium absorption. As the absorption coefficient increased, image quality improved. However, the best-matched absorption obtained with the $5\times$ objective was higher than that with the $1.25\times$ objective, indicating that higher objective NA required greater medium absorption to compensate for scattered light effects. Repeating the experiment with an objective lens of larger NA, it was found that the best-matched absorption still persisted, and the $25\times$ water immersion objective lens with a larger NA required a greater best-matched absorption for imaging.

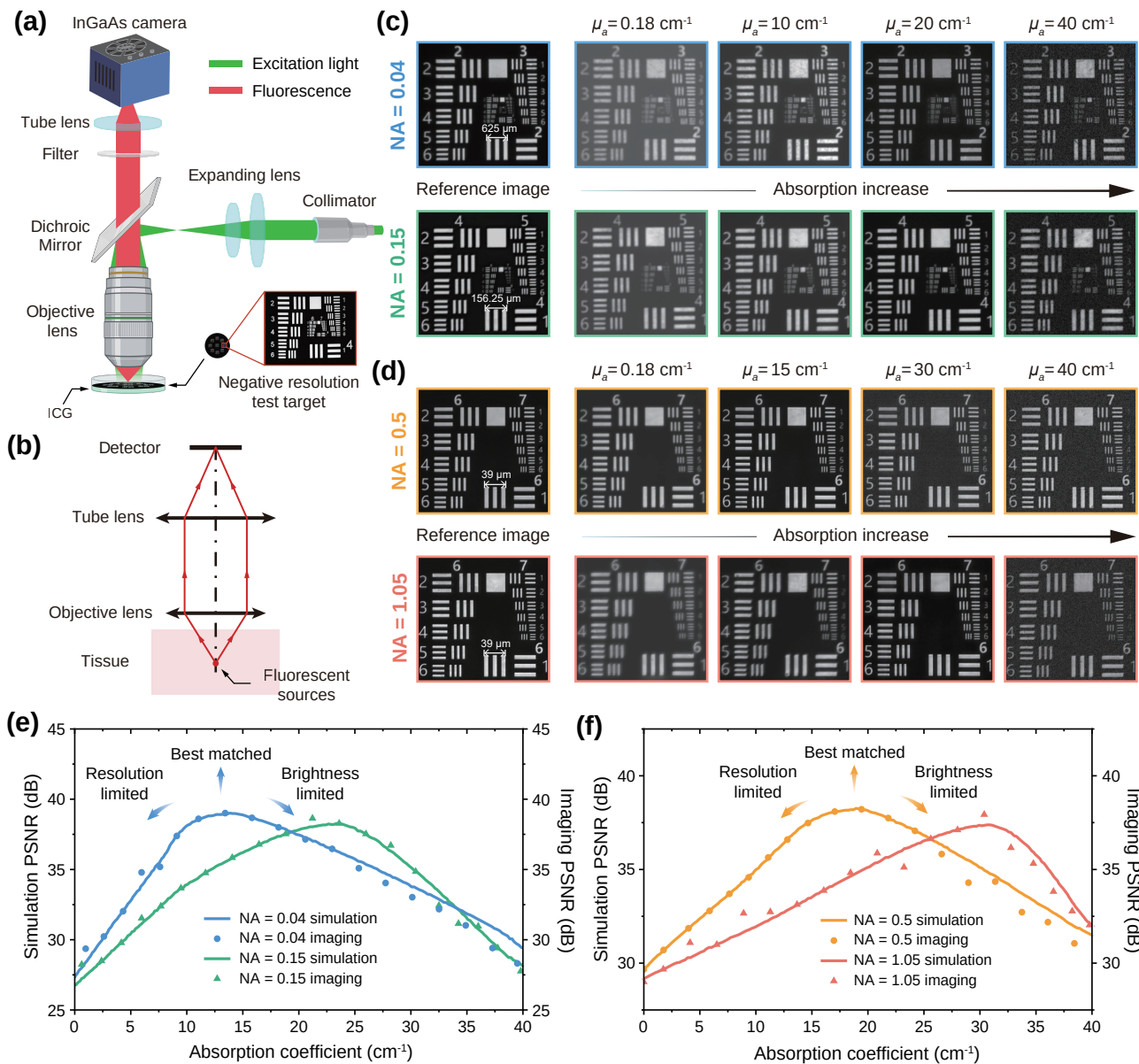


Figure 1 Best-matched absorption in fluorescence wide-field microscopic imaging. **a.** Schematic diagram of the NIR fluorescence wide-field microscopic imaging system for *in vitro* phantom imaging. **b.** Optical path diagram of fluorescence wide-field microscopic imaging simulation. Both the objective lens and the tube lens are simplified to ideal thin lenses for simulation. **c–d.** Fluorescence imaging through a negative resolution test target and 1 mm medium at low (1.25 $\times$, 5 $\times$) and high (20 $\times$, 25 $\times$) magnifications. The reduced scattering of the medium was fixed at 5 cm^{-1} , and the absorption increased from 0.18 cm^{-1} to 40 cm^{-1} . **e–f.** The relationship between the imaging PSNR and the medium absorption coefficient under different objective NA. The lines represent the simulation data, and the dots represent the *in vitro* phantom imaging data.

2.2 Influence of imaging system NA, imaging depth, and probe brightness on fluorescence image SBR

In fluorescence wide-field microscopy, variations in imaging parameters—including system NA, imaging depth, and probe brightness—significantly impact imaging outcomes. Therefore, analyzing the relationship between fluorescence image SBR and these variables is critical for optimal imaging window selection. We conducted simulations with reference to the optical properties of mouse skin tissue and obtained the best-matched absorption at different wavelengths. In the simulation, an ideal probe model was implemented with uniform fluorescence intensity across wavelengths. To visualize correlations between best-matched absorp-

tion and tissue optical parameters, we took reduced scattering coefficient as the independent variable and plotted the best-matched absorption spectra under varying imaging conditions. The actual tissue absorption spectra (black curves in Figure 2) were superimposed with best-matched absorption spectra, and the intersection points identified optimal imaging wavelengths. However, practical imaging collects broadband light rather than single wavelengths, while multiple factors influence image quality. Thus, identifying spectral ranges that provide favorable SBR under specific conditions is essential for defining optimal imaging windows.

When the NA of the imaging system increases, it collects

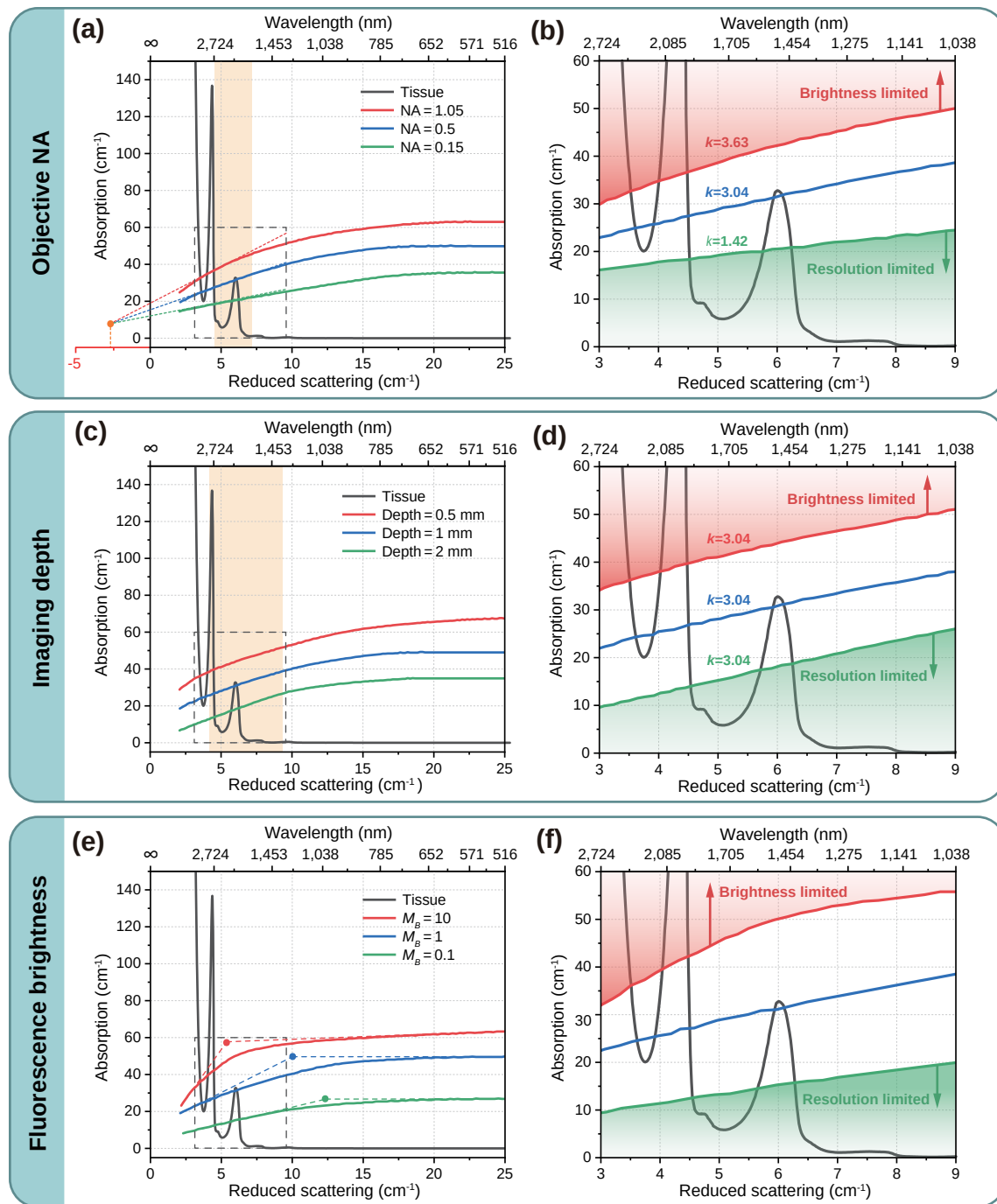


Figure 2 The influence of different imaging conditions on the SBR of fluorescence images. **a–b.** The best-matched absorption coefficient spectra of ideal probe in mouse skin for imaging under objective lens conditions of different NA; **c–d.** the best-matched absorption spectra of the ideal probe in mouse skin at different imaging depths; **e–f.** the best-matched absorption spectra of the ideal probe in mouse skin at different probe brightness. The yellow shadow indicates the range of the linear area. Figure 2b, d, and f is enlarged parts of the black dashed boxes in Figure 2a, c, and e. k represents the average slope of the curve in the linear region. The green shaded area indicates wavelengths where tissue absorption is below the best-matched curve; here, image quality is resolution-limited due to insufficient suppression of scattered photons. The red shaded area indicates wavelengths where tissue absorption exceeds the best-matched curve; here, image brightness is limited due to excessive signal attenuation. The optimal imaging window lies near the intersection of the tissue absorption spectrum (black curve) with the best-matched absorption curve.

photons over larger angles, enabling more fluorescence capture at identical excitation power densities. However, a larger collection angle also increases collection probability for multiply scattered photons, elevating background noise. Therefore, greater absorption is required to suppress these scattered photons with longer

optical paths. The simulation conclusion indicated that the best-matched absorption increased with enhanced tissue scattering. As NA increased, the curve shifted upward, indicating that stronger absorption was required to achieve the best imaging quality, as shown in Figure 2a. Furthermore, within the reduced scattering

coefficient range of approximately $5\text{--}7\text{ cm}^{-1}$ (yellow region in Figure 2), the best-matched absorption curve exhibited linear dependence on scattering, with steeper slopes observed for higher-NA systems (Figure 2b). Beyond this range, the absorption growth rate decelerated, producing convex curvature. This nonlinear behavior may arise because excessive scattering requires higher absorption to achieve balance, which leads to a higher demand for probe brightness, but the probe brightness in the simulation is fixed. Therefore, when the medium scattering increases, best-matched absorption will not increase indefinitely.

When the actual absorption of tissue locates below the best-matched absorption curve (green shaded area in Figure 2), the resolution of the image will be limited due to strong scattering, whereas the wavelengths with absorption exceeding the best-matched absorption (red shaded area in Figure 2) are limited in image brightness due to excessive absorption. Based on the NA of the objective lens used during imaging, selecting the wavelengths corresponding to spectral intersections near the best-matched absorption curve can obtain high-SBR images. Notably, although wavelengths where tissue absorption exceeds the best-matched absorption are unsuitable for standalone imaging due to insufficient brightness, their characteristically high ballistic photon proportions enable combination with imaging windows centered near the best-matched absorption for imaging together. For instance, when imaging through mouse skin with an NA = 0.5 objective lens, resolution-limited regions (<1,400 and 1,600–1,850 nm) should be avoided. Both 1,500 nm bandpass and 2,000 nm long-pass (LP) constitute viable alternatives.

We next investigated the relationship between imaging SBR and the depth of the imaging target. The best-matched absorption decreased with larger imaging depth when only the imaging depth was modified (Figure 2c and d). As imaging depth increases, the optical path for the transmission of fluorescent photons in the tissue extends, intensifying absorption and scattering by the tissue. This reduces the number of photons penetrating the tissue, resulting in underexposure of the imaging. Within the reduced scattering coefficient range of $5\text{--}7\text{ cm}^{-1}$, best-matched absorption exhibited linear dependence on scattering. At fixed probe brightness and objective NA, the slopes remained consistent across imaging depths (Figure 2d), implying an overall downward shift in the absorption “acceptable” for optimal imaging as depth increased.

Probe brightness is also an important factor affecting the imaging quality. To evaluate its impact on best-matched absorption, we used the fluorescence intensity of ICG solution as a reference ($M_B = 1$; see Sect. 5.2), and conducted simulations at probe brightness of $M_B = 0.1$, 1, and 10 under otherwise identical conditions. Results demonstrated significant brightness dependence: higher-brightness probes corresponded to elevated best-matched absorption (Figure 2e and f). Notably, at low scattering levels, high-brightness probes exhibited pronounced absorption increases with rising scattering coefficients. This sensitivity implies that when long-wavelength probes with higher brightness emerge in the future, precise imaging windows selection will be required as minor spectral adjustments may substantially impact image quality. Conversely, lower-brightness probes necessitate broader imaging windows to include wavelengths with lower tissue absorption, ensuring a relatively high imaging SBR.

2.3 Best-matched absorption for ICG-based fluorescence wide-field microscopic imaging

The preceding section analyzed wide-field microscopic imaging with an ideal probe exhibiting uniform emission. Since actual probes display wavelength-dependent emission intensities, analysis on specific probe is essential. Here, we took ICG as an example to analyze the optimal imaging window when penetrating mice skin for imaging. At reference brightness ($M_B = 1$), ICG’s emission spectrum and the best-matched absorption spectrum (using a $5\times$ objective lens, NA = 0.15) are presented in Figure 3a. Although the fluorescence emission peak of ICG is near 850 nm, the mouse skin scattering coefficient exceeds 9 cm^{-1} at this wavelength (Figure S1 in Supplementary information), limiting the achievable SBR. Within the tissue’s reduced scattering coefficient range of $5\text{--}9\text{ cm}^{-1}$, an absorption peak intersects with the best-matched absorption spectrum. This spectral region was therefore prioritized for investigation.

Simulations generated best-matched absorption curves for ICG imaging through mouse skin under varied conditions (Figure 3b). Initially, using a $5\times$ objective lens, under the conditions of NA = 0.15, probe brightness $M_B = 1$, and an imaging depth = 1 mm, the best-matched absorption was obtained as the blue curve in Figure 3b, intersecting the tissue absorption spectrum at 1,237 nm. Reducing tissue thickness to 0.8 mm increased best-matched absorption, and redshifted the intersection to 1,332 nm (green curve). Subsequently, the objective lens in the simulation was changed with NA increased to 1.05, and the immersion medium was replaced with water (refractive index $n = 1.33$). The best-matched absorption rose steeply (yellow curve), intersecting tissue absorption at 1,380 nm. This indicates that enhanced system NA significantly increases scattered light collection, necessitating stronger tissue absorption for compensation. Finally, increasing probe brightness to $M_B = 10$ dramatically elevated best-matched absorption (red curve), shifting the intersection to 1,401 nm. Thus, wavelengths $\geq 1,400\text{ nm}$ constitute the optimal imaging window under these conditions.

To validate the simulation results, we performed *in vitro* phantom imaging experiments using ICG. Fluorescence wide-field microscopy was conducted using a $5\times$ objective (NA = 0.15) with a 900 nm LP filter, penetrating a scattering medium equivalent to 1 mm of mouse skin. The obtained fluorescence images (red channel) were merged with reference images (cyan channel), as shown in Figure 3c. Significant scattered-light background was observed in the image. After replacing the filter in front of the camera with 1,200 LP, the scattered background of the image was weakened and the image SBR increased (Figure 3d). Subsequent reduction of medium thickness by 0.2 mm substantially diminished noise in dark image regions, though minimal scattered-light noise persisted (Figure 3e). After further substituting the filter with 1,300 LP, the background noise additionally suppressed (Figure 3f), which was consistent with the simulated best-matched absorption at 1,332 nm. Quantitative analysis of PSNR demonstrated progressive improvement from 37.2 to 39.6, 42.1, and 44.1 across these experimental steps (Figure 3g), confirming that both longer-wavelength imaging and reduced medium thickness enhance fluorescence image quality.

For comprehensive validation, experiments with a high-power

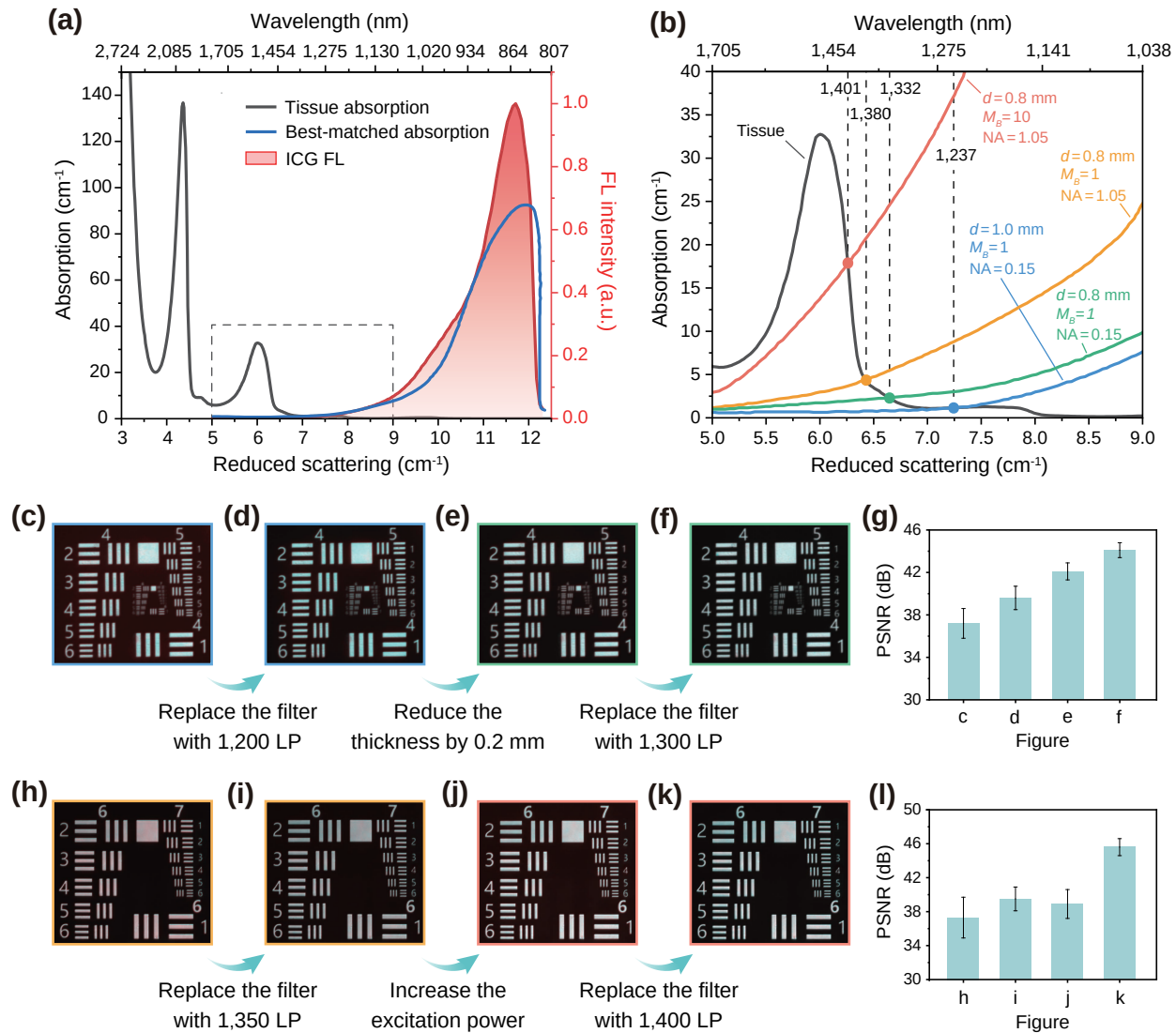


Figure 3 Selection of fluorescence imaging window when using ICG as the probe. **a.** The absorption spectrum of the tissue (black, measured from mouse skin), the emission spectrum of ICG (red), and the best-matched absorption spectrum of the tissue (blue). The reduced scattering coefficient (μ'_s) shown on the lower axis corresponds to the measured values from mouse skin. **b.** The best-matched absorption spectra of ICG through fluorescence microscopy of mouse skin under different imaging conditions. d represents the imaging depth, M_B represents the probe brightness magnification, and NA represents the numerical aperture of the objective lens. **c–f.** Fluorescence microscopic imaging of ICG through a negative resolution target and medium under a 5 $\times$ objective lens when various imaging conditions are changed. **g.** Imaging PSNR analysis of Figure 3c–f. The data from $n = 3$ imaging experiments were used for analysis, and presented as mean $\pm$ SD. **h–k.** Fluorescence microscopic imaging of ICG through a negative resolution target and medium under a 25 $\times$ objective lens when various imaging conditions are changed. **l.** Imaging PSNR analysis of Figure 3h–k. The data from $n = 3$ imaging experiments were used for analysis, and presented as mean $\pm$ SD. The images in Figure 3c–f and h–k are formed by superimposing the reference image (cyan) and the fluorescent image passing through the medium (red).

objective (25 $\times$, water immersion, $NA = 1.05$) were also conducted. After replacing the objective lens and matching the excitation light power density, the PSNR of the obtained image is 37.3 under 1,300 nm LP filter. The increased NA elevated best-matched absorption requirements, shifting the intersection with tissue absorption to longer wavelengths (1,380 nm). As expected, filter replacement with a 1,350 nm LP filter significantly improved image quality (Figure 3i, PSNR = 39.5). To exploit lower-scattering wavelengths for further PSNR enhancement, probe brightness requires augmentation. Increasing power density to 150 mW/cm^2 ($M_B = 10$) and switching the imaging window to

1,400 LP, the PSNR of the imaging increased to 45.6 (Figure 3k). These results align with simulations: higher- NA objectives necessitate longer-wavelength imaging for PSNR improvement, due to the angle at which the system collects light increases. However, excessive excitation power may reduce PSNR due to enhanced collection of multiply scattered photons in the tissue.

2.4 The selection of the imaging window when existing other fluorescent targets outside the depth of field

The DOF in a fluorescence wide-field microscope is inherently

limited, making the system susceptible to interference from fluorescent targets located outside the focal plane. As our simulations consider only geometric optics processes without taking into account the diffraction-limited DOF, the geometric DOF_{geo} is adopted as the effective DOF in the following analysis. For systems with a relatively low NA, this DOF can be expressed by Equation (1):

$$\text{DOF} \approx \text{DOF}_{\text{geo}} = \frac{\Delta n \cdot f_{\text{obj}}}{2\text{NA} \cdot M}, \quad (1)$$

where Δ represents the pixel size; n is the refractive index of the immersion medium; the system magnification $M = \frac{f_{\text{tube}}}{f_{\text{obj}}}$, f_{tube} is the focal length of the tube lens, and f_{obj} is the focal length of the objective lens. As indicated by the equation, DOF is directly proportional to the detector pixel size. For instance, with the 5× objective (NA = 0.15) and a detector pixel size of 15 μm used in our simulations, the calculated DOF is 360 μm. Using the optical properties of mouse brain tissue as reference, we conducted simulations to investigate three common situations involving out-of-focus fluorescent interference: (1) Signal obstruction from targets shallower than the focal plane. (2) Background interference from targets deeper than the focal plane. (3) Partial blurring due to the physical extent of the fluorescent target itself exceeding the system's DOF. This section analyzes the optimal imaging window selection for each scenario.

When fluorescence signals exist shallower above the focal plane of the objective lens (Figure 4a), they lie outside the DOF and

diffuse into blurred spots at the detector. Due to their shorter pathlength through tissue, these superficial signals undergo less scattering and absorption than the in-focus target, leading to a pronounced impact on image quality. Figures 4b–e simulate a fluorescent target at 0.5 mm depth within mouse brain tissue, with interfering signals at shallower depths. When the defocus of the object is 150 μm (less than the close-up depth of the system), all fluorescence signals are clear, as shown in Figure 4b. Here, the shallower target exhibits higher brightness due to greater excitation light intensity. As defocus increases, the superficial signal gradually diffuses into a blurred spot with diminishing average intensity (Figures 4c and d). At a large defocus of −480 μm, the signal fully disperses into background noise (Figure 4e).

Imaging of superficial biological structures is often compromised by deeper background fluorescence (Figure 4f). For example, during fluorescence imaging of subcutaneous abdominal blood vessels in mice, signals from deeper organs like the liver or intestines can contribute to the background. Owing to the limited DOF, these out-of-focus deeper fluorescence particles form diffuse patterns in the image, with larger defocus values producing more extensive blurring (Figures 4h–j). Unlike superficial signal interference, deep background signals experience greater tissue scattering and absorption. Especially when significantly defocused, the fluorescence energy spreads over a larger area, thereby exerting a relatively weaker influence on the in-focus target.

The physical size or spatial distribution of a fluorescent target may itself exceed the system's DOF, causing partial blurring of the image (Figure 4k). A representative example is cerebrovascular

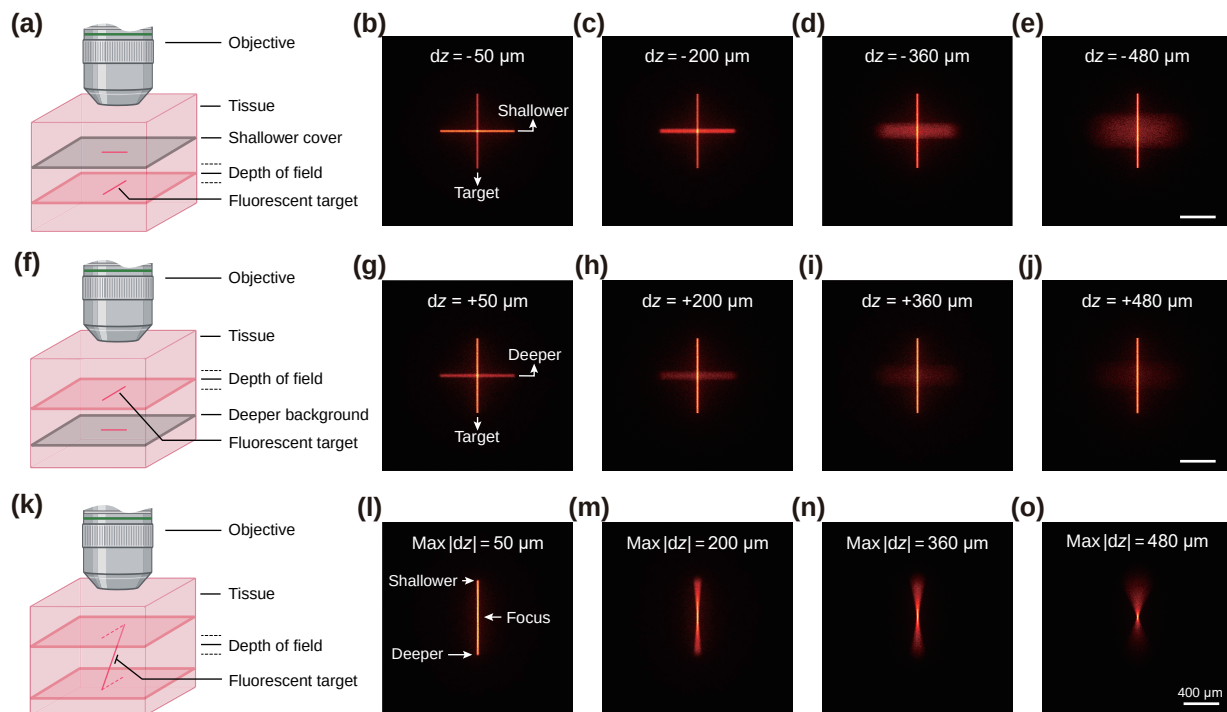


Figure 4 Simulation of fluorescent targets outside the DOF of the system. **a.** Schematic diagram of fluorescence signal occlusion in the shallow layer of the fluorescent target; **b–e.** simulation images with different defocus amounts of shallow occlusion signals; **f.** schematic diagram of the presence of fluorescence signal background in the deep layers of the fluorescent target; **g–j.** simulation images with different defocus amounts of deep background signals; **k.** schematic diagram of the axial depth of the fluorescent target exceeding the DOF of the system; **l–o.** simulation images of different fluorescent targets with the maximum defocus.

imaging, where longitudinally oriented vessels may extend beyond the focal volume. To simulate this scenario, we rotated the fluorescent target around its center toward the optical axis, causing the edge of the object to gradually exceed and move away from the DOF of the system (Figures 4l–o). Simulations were performed with maximum defocus values of 50, 200, 360, and 480 μm . At 200 μm maximum defocus, the image exhibits symmetrical blurring (Figure 4m). As the maximum defocus increases to 480 μm (Figure 4o), the deeper edges of the object appear dimmer due to stronger tissue absorption and scattering, while the upper regions of the image (i.e., the shallower defocus point of the target) remain brighter. Furthermore, increased defocus toward the edges causes points to blur into larger spots, concurrently reducing the average fluorescence intensity.

Next, we performed simulations across wavelengths from 900 nm to 2,500 nm for each of the three scenarios. Using images without shallow or deep fluorescence interference as reference images, we quantitatively evaluated the PSNR of the acquired images. In the presence of superficial signal interference, a larger defocus distance $|dz|$ of the shallow signal resulted in a higher imaging SBR, as shown in Figure 5a. The PSNR curve exhibited notable dips in regions of strong absorption, such as $\sim 1,450$ and $1,900$ nm, where high tissue absorption adversely affected the imaging of deeper targets. Excessive absorption led to underexposure and a rapid decline in PSNR. Owing to reduced optical scattering at longer wavelengths, the PSNR curve generally increased with wavelength, reaching a maximum near $1,700$ nm. Thus, in the presence of superficial out-of-focus interference, using the wavelengths around $1,700$ nm is preferable. Superficial defocused targets—especially those just beyond the system’s DOF—strongly influence image quality due to higher excitation intensity and shorter emission pathlengths. Therefore, the optimal strategy is to maximize the system DOF to include such interfering targets within the focal volume. As imaging depth increased, the PSNR decreased (Figure 5b). This decrease was particularly pronounced in shorter wavelengths (900 – $1,300$ nm), while the $1,700$ nm band maintained relatively high PSNR across all depths.

For deep background interference, image quality remained high at large defocus distances ($dz = +480$ and $+360$ μm). However, when the defocus was only $+200$ μm —just beyond the DOF—background fluorescence significantly degraded image quality, causing a sharp decrease in PSNR (Figure 5c). The PSNR curve also increased overall with wavelength and displayed three local maxima near $1,450$, $1,890$, and $2,200$ nm, which correspond to wavelengths with strong light absorption. This indicates that light absorption can substantially improve image quality under deep background interference. A prominent PSNR peak occurred near $1,890$ nm, followed by a rapid decrease at longer wavelengths due to extremely high absorption, which limited the number of detectable fluorescence photons. Within the 900 – $2,500$ nm range, the highest PSNR was observed in the third near-infrared (NIR-III, $2,080$ – $2,340$ nm) window, which combines low scattering with moderate absorption. It is worth noting that with the development and commercialization of detectors with longer corresponding wavelengths (such as mercury cadmium telluride detectors), the NIR-III window is expected to be applied in biomedical imaging and highlight its high SBR advantage. With increasing imaging depth, PSNR decreased overall (Figure 5d),

and the benefit conferred by optical absorption gradually diminished.

When the physical size of an object exceeds the system’s DOF, the resulting image blur persists even in the absence of tissue scattering and absorption. Therefore, reference images for PSNR analysis must account for the defocused portions, requiring separate simulations for each combination of imaging depth and maximum defocus. The resulting PSNR curves are shown in Figure 5e. Overall, PSNR decreased with increasing defocus. When the target center was at 0.5 mm depth, local PSNR maxima occurred in wavelengths with higher tissue absorption (Figure 5e and the black curve in Figure 5f). However, at greater imaging depths, strong absorption bands such as $\sim 1,450$ nm suffered from insufficient signal, causing a rapid PSNR drop (blue and purple curves in Figure 5f), yielding curves similar in shape to those in Figure 5a. In such cases, the optimal imaging window should be selected according to the actual depth of the target.

3. Discussion

Fluorescence wide-field microscopy serves as a valuable tool in applications such as cerebrovascular imaging and precise tumor analysis [26–29,31,32]. In this study, we employed ICG as a reference probe and systematically investigated the selection of imaging windows in the NIR range by combining fluorescence patterns generated with a negative contrast resolution target with comprehensive imaging simulations. Our findings indicate that, similar to macroscopic imaging based on photographic systems, there also exists a best-matched absorption coefficient in fluorescence wide-field microscopic imaging. Imaging within the spectral window adjacent to the wavelength corresponding to this coefficient yields the optimal SBR. Different from photographic imaging systems, imaging system NA has a great influence on microscopic imaging. As the NA increases, the system’s fluorescence collection efficiency improves; however, this comes at the cost of collecting more scattered photons, thereby necessitating higher tissue absorption to counteract scattering-induced background noise, resulting in an elevation of the system’s best-matched absorption coefficient. Additionally, under conditions of sufficient fluorescence target brightness, the best-matched absorption spectrum exhibits a linear relationship with the tissue scattering spectrum, with the slope of this relationship being influenced by the NA of the imaging system.

By integrating simulation results with experimental ICG fluorescence imaging, we observed that increases in fluorescence target brightness, reduction in imaging depth, and higher system NA collectively shift the optimal imaging window for ICG from above $1,200$ nm to beyond $1,400$ nm, accompanied by a corresponding improvement in the SBR. Furthermore, this study addressed three typical defocus scenarios in microscopic imaging—superficial defocus obstruction, deep defocus background, and local target defocus—and analyzed the corresponding imaging window selection strategies. We demonstrated that in superficial obstruction scenarios, increased optical absorption does not enhance image quality, and longer wavelengths with lower scattering (e.g., around $1,700$ nm) are preferable to reduce the influence of shallow scattered signals. In contrast, in the presence of a deep defocus background, absorption significantly improves image quality; wavelengths with moderate to high tissue

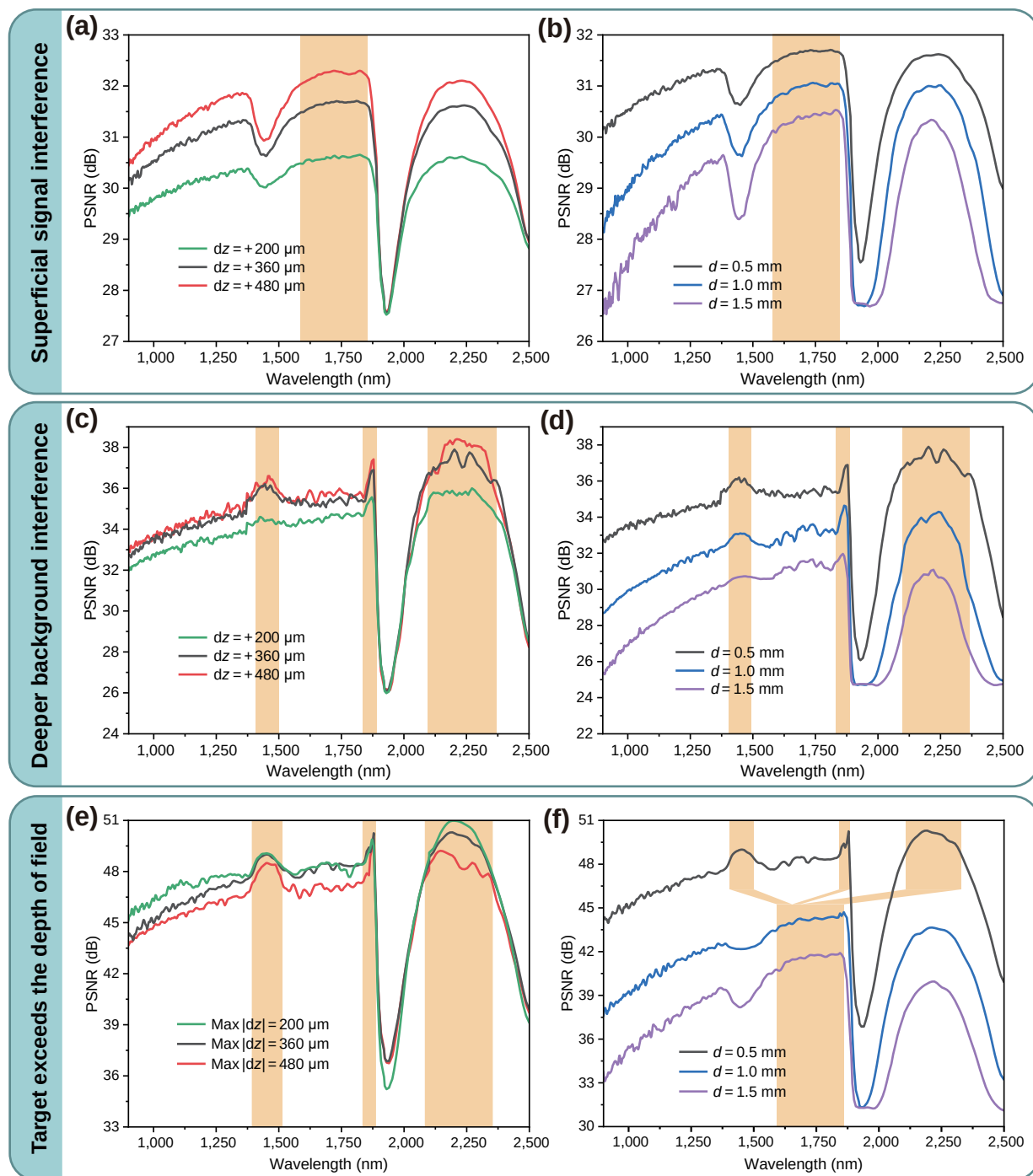


Figure 5 Image quality analysis and window selection when existing fluorescent targets are outside the system DOF. **a.** PSNR analysis of imaging under shallow occlusion with different defocus amounts at an imaging depth of 0.5 mm; **b.** PSNR analysis of images at different imaging depths when the system has shallow occlusion with a defocus of $-360\ \mu\text{m}$; **c.** PSNR analysis of deep backgrounds with different defocus amounts at an imaging depth of 0.5 mm; **d.** PSNR analysis of images at different imaging depths when the system has a deep background with a defocus of $+360\ \mu\text{m}$; **e.** imaging PSNR spectra under different maximum defocus amounts at an imaging depth of 0.5 mm; **f.** PSNR analysis of imaging at different depths when the maximum defocus is $360\ \mu\text{m}$. The yellow area indicates the wavelength range with better imaging quality under this condition.

absorption (e.g., near 1,450 or 2,200 nm) can effectively suppress the deeper background fluorescence. When the fluorescent target itself extends beyond the DOF, the choice of imaging window depends on the target depth: for superficial targets, higher-absorption bands (e.g., $\sim 1,450$ and $\sim 2,200$ nm) help maintain contrast without diminishing the benefit of absorption; for deep

targets, however, high-absorption bands are more likely to lead to underexposure of the defocused portions, and lower-scattering windows (e.g., around 1,700 nm) are recommended.

When selecting an imaging window for fluorescence imaging, it is essential to choose not only high-quality filters and dichroic mirrors, but also to account for the transmittance of optical

components such as the objective lens, as well as the response characteristics of the detector. These factors impose additional constraints on the imaging window. For instance, when performing NIR-II imaging with ICG, it is recommended to use a highly sensitive indium gallium arsenide (InGaAs) detector along with an objective lens and tube lens featuring infrared anti-reflection coatings. What's more, although the emission spectrum of a probe may vary with its microenvironment, the proposed framework for determining the best-matched absorption remains applicable, as it mainly depends on tissue optical properties and system parameters.

It is important to acknowledge certain limitations of our work and aspects that warrant further research. In terms of simulation, our model incorporated the interaction between fluorescent photons and tissue, their emission from the tissue surface, propagation through the imaging system, and eventual collection by the detector. However, it did not account for interactions between excitation light and tissue, and photon transport was described using geometric optics methods alone. As a result, our approach could not simulate scenarios involving polarized or structured illumination, nor could it accurately simulate more complex situations such as the reabsorption of emitted fluorescent photons or non-uniform fluorescence intensity caused by out-of-focus fluorophore absorption. Nevertheless, since practical imaging generally involves collecting light within a spectral range rather than at a single wavelength—which would lead to severe underexposure—it is sufficient to select an imaging window near the best-matched absorption, without requiring its precise value.

Furthermore, while we investigated the influence of various factors on the best-matched absorption through simulations and validated the findings using ICG with several commonly used objectives, further experiments are needed to generalize these conclusions to other fluorescent probes, imaging systems with different NAs, and more complex biological imaging contexts.

Although fluorescence wide-field microscopy offers a large field of view and high imaging speed, it inherently lacks sectioning capability compared to techniques such as confocal or two-photon fluorescence microscopy [33,34]. The method proposed in this study, by optimizing the imaging window, can minimize interference from out-of-focus signals; however, it still falls short of providing three-dimensional resolution. Combining deep learning approaches—for instance, training models to learn from confocal microscopic images [35]—may help combine the advantages of both imaging modalities. Moreover, for techniques such as light-sheet microscopy, confocal fluorescence microscopy, and photoacoustic imaging, where excitation characteristics and light–tissue interactions are more complex and critically influence image quality, the selection of the optimal imaging window remains an open question for future research.

4. Conclusions

In summary, this study introduces the concept of best-matched absorption in fluorescence wide-field microscopy and systematically investigates its relationship with imaging depth, system NA, probe brightness, and defocus conditions. The findings provide a practical strategy for selecting the optimal imaging window in area-array-detector-based fluorescence wide-field

imaging. This approach allows for convenient pre-selection of suitable imaging wavelengths, requiring only minimal fine-tuning during experiments to achieve images with the highest SBR. We believe this method will provide great help in future fundamental research and clinical imaging applications.

5. Methods

5.1 Construction of fluorescence wide-field microscopy imaging system

A cage-based fluorescence wide-field microscopy system was constructed as illustrated in Figure 1a. A 793 nm continuous-wave laser was output through an optical fiber, collimated using a large-aperture collimator (F810FC-780, Thorlabs), and passed through a beam-shaping lens group. The beam was then reflected downward by a dichroic mirror (900 nm long-wave pass) in a diverging state and entered the objective lens, where the excitation beam was focused at its front focal point. The shaped excitation beam overfilled the objective entrance pupil to ensure uniform illumination across the entire field of view. The objective and tube lens formed an infinitely conjugate system, focusing the image onto the detection surface of an InGaAs camera (SD640, Tekwin System). During imaging, the camera operated in the automatic exposure mode, and the gain remained constant.

Four fluorescence imaging objectives with different magnifications and NA were used in the experiments: PLAPON1.25X (1.25 $\times$, f = 180 mm, NA = 0.04, air immersion, working distance (WD) = 5 mm), MPLFLN5X2 (5 $\times$, f = 36 mm, NA = 0.15, air immersion, WD = 20 mm), UMLFLN20XW (20 $\times$, f = 9 mm, NA = 0.5, air immersion, WD = 3 mm) and XLPLN25XWMP2 (25 $\times$, f = 7.2 mm, NA = 1.05, water immersion, WD = 2 mm).

As shown in Figure S2 in Supplementary information, a negative contrast resolution target served as a fluorescence mask placed over a layer of ICG solution (0.1 mg/mL in dimethylsulfoxide (DMSO), thickness <0.5 mm). The remaining volume of the round-bottom glass dish above the resolution target could accommodate up to 10 mm of liquid medium. Intralipid (Baxter Healthcare Co., USA) and India ink (YuanYe Bio-Technology Co., China) were used to simulate scattering and absorption media, respectively. These were thoroughly mixed with water to form stable scattering and absorption media.

Fluorescence emitted by ICG penetrated the liquid medium, passed through the objective, was transmitted through the dichroic mirror and an emission filter (details listed in Table S1 in Supplementary information), and was finally focused onto the camera by the tube lens. The entire system was aligned using a 60 mm cage system to maintain co-axiality. When changing objectives, the beam-shaping lens group was fine-tuned to ensure illumination uniformity. The objective was mounted on a Z-axis motorized stage, while the imaging sample was placed on an X-Y translation stage.

5.2 Implementation of imaging simulation

The imaging simulation model consisted of two main stages: (1) photon propagation within tissue and emission, and (2) propagation through the optical system to the detector. For the

first stage, we employed a Monte Carlo method based on the MCML (Monte Carlo modeling of light transport in multi-layered tissues) algorithm developed by Wang et al. [36], which simulates light transport in radially symmetric multi-layered tissues. The algorithm was optimized to accommodate more flexible imaging scenarios. Fluorescent photons were emitted from specific depths and positions within the tissue at random angles. Each photon advanced by a random step size, followed by absorption or scattering events. Absorption reduced photon energy, while scattering altered the photon direction based on the medium's anisotropy coefficient. If the photon retained sufficient energy, a new step size was set and the process repeated. Terminated photons were replaced by new emissions until all photon propagation was complete. Photons reaching the tissue boundary underwent refraction or reflection, with those escaping the top surface proceeding to the second stage.

The wide-field microscopy system was simplified to an infinity-corrected magnification system comprising an objective and a tube lens, both modeled as ideal thin lenses (Figure 1b). The tube lens had a focal length of 180 mm (Olympus standard) and a diameter of 60 mm. System NA was controlled by adjusting the objective lens diameter, while magnification was varied by changing the objective focal length. Simulation parameters for the four objectives matched those described in Sect. 5.1.

Two-dimensional narrow lines served as fluorescence targets (Figure S3 in Supplementary information), with sizes varying by magnification: 4 mm × 0.2 mm (1.25×), 0.8 mm × 0.04 mm (5×), 0.2 mm × 0.01 mm (20×), and 0.16 mm × 0.008 mm (25×). Both photon number and weight (energy per photon) affected the represented fluorescence target brightness. We defined a luminance coefficient M_B representing the fluorescence intensity multiple relative to a reference ($M_B = 1$ corresponding to 0.1 mg/mL ICG in DMSO under 793 nm excitation at 20 mW/cm²). The baseline photon count per simulation was $N_0 = 1 \times 10^7$ with default weight $\omega_0 = 1$. For different luminance levels, both photon count and weight scaled linearly with M_B . That is, the number of photons used in each simulation is the product of the reference photon number N_0 and the luminance coefficient M_B . When no specific indication is given (as in Figures 1 and 5), $M_B = 1$.

5.3 Measurement of optical parameters

The optical parameters are tissue-specific and should be measured or obtained from reliable references. In this study, absorption and scattering coefficients of mouse skin tissue and liquid media were measured using an integrating sphere method for its high accuracy [37]. Measurements were performed using a spectroradiometer (Fieldspec 4, Analytical Spectral Devices-ASD, Boulder, CO USA) equipped with an integrating sphere (RTS-3ZC, Analytical Spectral Devices Inc.). The instrument provides spectral resolutions of 3 nm (350–1,000 nm) and 8 nm (1,000–2,500 nm).

The Kubelka-Munk (KM) theory [37] was applied to calculate the reduced scattering (μ'_s) and absorption (μ_a) coefficients. Diffuse reflectance (R) and transmittance (T_d) spectra were measured, and the coefficients were derived as:

$$\mu_a = \frac{1}{2}A_{KM}, \quad \mu'_s = \frac{4}{3}S_{KM}. \quad (2)$$

Among them, the KM coefficients A_{KM} and S_{KM} are given by:

$$S_{KM} = \frac{1}{yd} \cdot \ln \left[\frac{1 - R(x-y)}{T_d} \right], \quad (3)$$

$$A_{KM} = (x-1)S_{KM}, \quad (4)$$

where d represents the thickness of the sample, and x and y are calculated based on the following equation:

$$x = \frac{1 + R^2 - T_d^2}{2R}, \quad y = \sqrt{x^2 - 1}. \quad (5)$$

5.4 Calculation method of PSNR

The PSNR was employed to evaluate image fidelity by quantifying differences between reference and noisy images. As a full-reference quality metric, PSNR provides an objective and computationally efficient means for assessing large image sets. Higher PSNR values indicate better image quality, corresponding to a lower impact of the scattering effect on the image in this study. The noisy images were obtained by imaging through a scattering medium, while the reference image was acquired under the same imaging conditions without the presence of the scattering medium. For the reference image, both the absorption coefficient and the scattering coefficient are 0.

PSNR was calculated as:

$$\text{PSNR} = 10 \times \lg \left(\frac{2^n - 1}{\text{MSE}} \right), \quad (6)$$

where n is the bit depth of the image ($n = 8$ in this paper), and the mean squared error (MSE) is defined by the following equation:

$$\text{MSE} = \frac{1}{mn} \sum_{i=1}^m \sum_{j=1}^n |I(i, j) - I_0(i, j)|, \quad (7)$$

where m and n represent image dimensions; while I and I_0 correspond to the non-normalized intensities of the noisy image and the reference image, respectively.

5.5 Construction of the NIR fluorescence emission spectroscopy system

The emission spectrum of ICG was measured using a custom-built NIR fluorescence spectroscopy system (Figure S4 in Supplementary information). Excitation light was focused through a lens to laterally excite the fluorescent material in a cuvette. An objective lens (XLPLN25XWMP2, 25×, NA = 1.05) was positioned orthogonally to the excitation axis to collect fluorescence from the opposite side of the cuvette. The emitted light was collected by a large-aperture collimator (F810SMA-1310, Thorlabs) before being transmitted via optical fiber to a NIR spectrometer (NIR-2200, Idea Optics). To maximize detection efficiency and minimize noise, the excitation point was positioned close to the cuvette edge, with the collection path maintaining orthogonal alignment to the excitation axis.

Supplementary information

The supplementary information is available online at <https://doi.org/10.3724/PXLIFE.2025-0017>. The supplementary materials

are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.

Data availability

The data and materials of this study are available from the corresponding author upon reasonable request.

Authors' contributions Jun Qian and Tianxiang Wu conceived and designed the project. Tianxiang Wu performed the simulation imaging experiments. Baixuan Wu and Tianxiang Wu performed the data analysis and wrote the original manuscript. Baixuan Wu, Zhe Feng, and Jun Qian wrote and reviewed the final version of the text.

Compliance and ethics The author(s) declare that they have no conflict of interest.

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